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Short communication

Alternative oxidase 1 (*Aox1*) gene expression in roots of *Medicago truncatula* is a genotype-specific component of salt stress tolerance

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ABSTRACT

Alternative oxidase (AOX) is the central component of the non-phosphorylating alternative respiratory pathway in plants and may be important for mitochondrial function during environmental stresses. Recently it has been proposed that *Aox* can be used as a functional marker for breeding stress tolerant plant varieties. This requires characterization of *Aox* alleles in plants with different degree of tolerance in a certain stress, affecting plant phenotype in a recognizable way. In this study we examined *Aox1* gene expression levels in *Medicago truncatula* genotypes differing in salt stress tolerance, in order to uncover any correlation between *Aox* expression and tolerance to salt stress. Results demonstrated a specific induction of *Aox1* gene expression in roots of the tolerant genotype that presented the lowest modulation in phenotypic and biochemical stress indices such as morphologic changes, protein level, lipid peroxidation and ROS generation. Similarly, in a previous study we reported that induction of antioxidant gene expression in the tolerant genotype contributed to the support of the antioxidant cellular machinery and stress tolerance. Correlation between expression patterns of the two groups of genes was revealed mainly in 48 h treated roots. Taken together, results from both experiments suggest that *M. truncatula* tolerance to salt stress may in part due to an efficient control of oxidative balance thanks to (i) induction of antioxidant systems and (ii) involvement of the AOX pathway. This reinforces the conclusion that differences in antioxidant mechanisms can be essential for salt stress tolerance in *M. truncatula* and possibly the corresponding genes, especially *Aox*, could be utilized as functional marker.

Introduction

Soil salinity is one of the major abiotic stress factors that restricts arable land and reduces crop productivity worldwide. High salt concentration adversely affects plant growth and development inducing water deficit, ionic toxicity, nutrient imbalance, and oxidative stress (Munns and Tester, 2008). Salt tolerance is a complex genetic trait that shows all the characteristics of a multigenic trait, with quantitative trait loci (QTLs) identified in barley, citrus, rice, tomato and legumes (Rose, 2008; Frary et al., 2010).

Salt tolerant genotypes may reduce ionic toxicity, adjust their osmotic pressure by the synthesis of compatible solutes (Munns and Tester, 2008), enhance antioxidant mechanisms for efficient reactive oxygen species (ROS) scavenging (Miller et al., 2011), or use any combination of the above strategies that will effectively protect the plant from salinity stress. In a recent study, we examined antioxidant responses of *Medicago truncatula* genotypes differing in salt tolerance (Mhadhbi et al., 2011). Our results suggested that tolerance behaviour could be related to the induction of antioxidant genes in plant roots, leading to more efficient enzyme stimulation and protection. Following, the response of alternative oxidase (AOX) was also examined in the same genotypes under salt stress. AOX is a key enzyme in the respiratory chain of plants. It mediates the cyanide-resistant pathway that branches from the main respiratory chain at the level of ubiquinone (Florez-Sarasa et al., 2009; Gupta et al., 2009). Plant AOXs are encoded by a small,

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nuclear multi-gene family. *Aox* expression patterns display variability and typically *Aox* genes fall into two discrete subfamilies, *Aox1* and *Aox2*, the former being present in all plants and the latter restricted in eudicot species (Vanlerberghe et al., 2009). Generally, the *Aox1*-type genes are induced by many different kinds of stress, whereas *Aox2*-type genes are expressed in a constitutive or developmentally regulated way (Polidoros et al., 2005, 2009). The AOX pathway may protect against harmful ROS generation. It controls the mitochondrial generation of ROS by stabilizing the reduction state of electron transport. AOX is an enzyme that allows plants to cope with environmental stress and it has been shown to play a role in salinity stress. In *Arabidopsis thaliana*, salt stress resulted in increased transcription levels of the AOX-encoding *Ataox1a*, along with the alternative NADH dehydrogenases *Atndb2* and *Atndb4* genes, indicating the formation of an abridged non-phosphorylating electron transport chain in response to salinity stress (Smith et al., 2009). In addition, plants constitutively over-expressing *Ataox1a*, with increased AOX capacity, showed lower ROS formation, 30–40% improved growth rates and lower shoot Na^+ content compared with controls, when grown under salinity stress conditions (Smith et al., 2009). Salt stress was also responsible for upregulation of *Aox* transcript along with other antioxidant genes in pea that was accompanied with higher AOX protein accumulation in isolated mitochondria (Martí et al., 2011). The above data suggest that AOX upregulation may be a critical component for plant stress defence under high salinity. Nevertheless, studies examining the relative induction of AOX in salt tolerant vs. salt sensitive varieties are scarce with contradictory results. *Aox* gene expression in cowpea (*Vigna unguiculata*) is different between a salt-tolerant and a salt-sensitive cultivar (Costa et al., 2007). Specifically, NaCl treatment induced an *Aox2*-type gene expression in roots of the sensitive cowpea cultivar whereas expression remained steady in roots of the tolerant one. On the contrary, AOX showed higher abundance in a salt tolerant wheat variety in both control and salt-treated conditions compared with a salt sensitive variety (Jacoby et al., 2010). The aim of this work was to assess the significance of *Aox1* gene expression in establishment of salt tolerance, as assessed from *Aox1* responses to salt treatments, in contrasting tolerant/sensitive genotypes of the model legume *M. truncatula*.

Materials and methods

Biological materials, culture conditions and biochemical analyses

The study was conducted using three *Medicago truncatula* genotypes: Jemalong (A17), a reference line with moderate sensitivity to salt, and two Tunisian local lines: TN6.18, a salt sensitive and TN1.11, a salt tolerant cultivar (Lazrek et al., 2009). Plants growing for 40 days on compost soil – perlite (3:1) mix were treated with 0 mM (c: control), 150 mM and 300 mM NaCl solution for 24 and 48 h.

Protein extraction and determination was performed according to Bradford method (1976) Lipid peroxidation was assayed using the thiobarbituric acid (TBARS) method (Singh et al., 2007). Hydrogen peroxide content was spectrophotometrically measured using potassium iodide solution (Chakrabarty et al., 2009).

Real time PCR gene expression analysis

Aox1 gene expression in roots and leaves of *M. truncatula* genotypes was analyzed by quantitative real-time RT-PCR (qRT-PCR) using a pair of *Aox1*-type specific primers with sequences: Aox1F: 5′-TTGGATCGAAGATGATGATGAG-3′/Aox1R: 5′-ACCAGCACCACCCTGAGAC-3′, designed based on the sequence of an *Aox1*-type EST of *M. truncatula* (GenBank Accession No. BG648214). Methodology of RNA isolation, cDNA synthesis, qRT-PCR, relative quantification of gene expression and statistical analysis was described in Mhadhbi et al. (2011).

Results

Phenotypic and biochemical responses to NaCl stress

Stress symptoms were observed after 48 h of 300 mM NaCl treatment and were manifested as leaf wilting in A17 and deformation of leaves in TN6.18 (data not shown). Wilting was also observed in stems of the sensitive TN6.18 genotype. Contrarily, no symptoms were detected in roots of all genotypes. Biochemical effects of salt stress were analyzed estimating hydrogen peroxide generation (ROS), total protein level, and MDA content (indicative of lipid peroxidation levels) in roots and shoots of the three genotypes. Results showed that H_2O_2 generation was significantly increased by salt treatment in leaves and mainly roots of TN6.18 genotype. For A17, H_2O_2 level was faintly increased mainly after 48 h. Changes on H_2O_2 generation were not significant in roots and leaves of TN1.11 genotypes (Table 1). The 24 h NaCl treatment had no significant effect in protein and MDA content of roots in all genotypes. On the other hand, 48 h of NaCl treatment resulted in decrease of protein content in roots of A17 and TN6.18 but did not affect protein level in the tolerant TN1.11 genotype (Table 1). MDA content was not affected in roots of TN1.11 while it increased in roots of A17 under 300 mM NaCl and in roots of TN6.18 under both 150 and 300 mM NaCl (Table 1). Protein content was significantly decreased after 48 h of salt treatment in shoots of the sensitive TN6.18 genotype. On the contrary, shoot MDA content increased after 24 and 48 h under both NaCl concentrations in all genotypes. This increase was more pronounced in leaves of A17 and TN6.18 (Table 1).

Aox1 gene response to NaCl stress

Aox1 expression was different between roots and leaves. In leaves, *Aox1* was slightly induced in response to 150 mM NaCl for 24 h in all genotypes, as well as for 48 h, except in A17 where a decrease was observed. Treatment with 300 mM NaCl for 24 h induced *Aox1* expression moderately inTN1.11 and highly in A17, while TN1.11 expression was slightly higher than the control and A17 expression was induced at the highest level observed in this study after prolonged (48 h) treatment. On the other hand, *Aox1* expression decreased dramatically in line TN6.18 after 24 h exposure while it increased in response to 48 h treatment with 300 mM NaCl.

In roots, *Aox1* gene expression initially increased in response to 150 mM NaCl for 24 h in all the genotypes, while it subsequently decreased in A17 and TN6.18 after 48 h of exposure. Interestingly, the decrease of *Aox1* expression in roots of A17 was concomitant with that of leaves, while this was not the case for the sensitive line TN6.18 (Fig. 1). It is noteworthy that induction of *Aox1* was remarkable in roots of the tolerant line TN1.11 at 150 mM NaCl after prolonged (48 h) exposure. At 300 mM NaCl, *Aox1* expression was increased in roots of TN1.11 and A17 at both time points, while it was initially increased and then strongly suppressed in TN6.18. Interestingly, the opposite expression pattern was observed in TN6.18 leaves (Fig. 1). Therefore, *Aox1* induction in roots correlated with the tolerance level of the different *M. truncatula* genotypes. Indeed, *Aox1* was highly induced in roots of the tolerant genotype TN1.11, a moderate induction was observed in the moderately tolerant A17 except for an initial slight repression at 300 mM NaCl, while an initial induction was observed in the most sensitive genotype TN6.18 which was followed by significant suppression after prolonged (48 h) NaCl application.